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AN INVESTIGATION OF THE HETEROGENEITY
OF ISOLATES OF *Mycoplasma ovipneumoniae*
USING RESTRICTION ENDONUCLEASE ANALYSIS.

A THESIS PRESENTED IN PARTIAL FUFILMENT OF
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NICHOLAS GRANT NORMAN

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ABSTRACT

Previous studies of *Mycoplasma ovipneumoniae* by Restriction Endonuclease Analysis (REA) (Mew, 1982) indicated that the species shows exceptional heterogeneity as compared to other species of pathogenic Mycoplasmas. This thesis further investigates this heterogeneity.

To get confirmation of the heterogeneity of *M. ovipneumoniae*, sixty isolates derived from three sheep on each of twenty farms, were examined by REA. All twenty independent isolates (i.e. isolates originating from sheep on different farms) gave REA patterns that were markedly different, with at most, only 5% of bands in common. Isolates from sheep on the same farm were found to be either indistinguishable, similar (i.e. at least 95% of bands in common) or markedly different (i.e. less than 5% of bands in common). Having confirmed the heterogeneity of *M. ovipneumoniae* isolates from sheep on different farms further study was directed at providing an explanation for this heterogeneity.

The stability of the *M. ovipneumoniae* genome was investigated by serial passage of a multiply cloned isolate *in vitro*. Three REA patterns, A, B and C (pattern A was the original pattern) were observed. These pattern changes were non-random in that they were reversible. Thus it appears that an internal rearrangement system is present in *M. ovipneumoniae*. No non-reversed REA pattern changes were seen. It was concluded that the pattern changes seen after serial *in vitro* passage were minimal, and that genomic instability could not explain the heterogeneity seen in *M. ovipneumoniae*.

Changed REA patterns must represent DNA changes which in turn may mean changes in proteins. To attempt to detect protein changes, 3 clones which showed patterns A, B and C respectively were examined by SDS-Polyacrylamide gel electrophoresis of total cellular proteins. No differences were detected. There remains the possibility that antigenic changes occurred

which might not be demonstrable by this method.

A second possible explanation for the heterogeneity seen in *M. ovipneumoniae* is that frequent genetic interchange between initially distinct REA strains might result in the generation of many new REA types that differ markedly from both parental strains. Three approaches were taken to investigate this possibility:

1. "Classical crosses" detected by antibiotic resistance markers.
2. Mixtures of two cultures of *M. ovipneumoniae* with different REA patterns were mixed and propagated together.
 - (a) Clones were selected from a mixed culture after it had been passaged for about 30 generations and examined by REA.
 - (b) "Presumptive recombinants", i.e. clones of *M. ovipneumoniae* which were resistant to two antibiotics, recovered from mixtures of singly resistant clones were examined by REA.
3. *M. ovipneumoniae* was examined for the presence of extra-chromosomal DNA which, if present, could facilitate genetic interchange.

Using these three approaches, we were unable to demonstrate genetic interchange in *M. ovipneumoniae* so it is unlikely that genetic interchange accounts for the considerable heterogeneity seen in the species.

It was concluded that the heterogeneity seen in the species is due to the presence of a large number of strains that are genetically stable with respect to REA, which have evolved over a long time period and which are independantly maintained.

We estimated the minimum number of strains of *M. ovipneumoniae* that must exist in a population so that when 29 independent isolates are examined, all will be different. With 95% certainty, this minimum number is 150.

The possibility that at least 150 *M. ovipneumoniae* strains could be maintained in New Zealand was discussed. By applying general epidemiological principles to *M. ovipneumoniae*, we concluded that many more than 150 could be independently maintained.

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CONTENTS

	<u>Page</u>
TITLE PAGE	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	v
LIST OF CONTENTS	vi
LIST OF FIGURES	xi
LIST OF TABLES	xiv
INTRODUCTION	1
CHAPTER 1: <u>HISTORICAL REVIEW</u>	3
1.1 Chronic Non-Progressive Pneumonia	3
1.11 Occurrence of Chronic Non-Pro- gressive Pneumonia	3
1.12 Economic importance of Chronic Non- Progressive Pneumonia	3
1.13 Aetiology of Chronic Non-Progressive Pneumonia	4
1.14 Transmission studies with Chronic Non-Progressive Pneumonia	5
1.2 Restriction Endonucleases	7
1.21 Discovery of characterisation of restriction endonucleases.	7
1.22 Class 1 Restriction Endonucleases	8
1.23 Class 2 Restriction Endonucleases	8
1.24 Nomenclature of Restriction Endon- ucleases	9
1.25 Specificity of class 2 Restriction Endonucleases	10
1.26 Applications of class 2 Restriction Endonucleases	10
a) Restriction Endonuclease mapping of DNA	10
b) Cloning of genes	11
c) Classification and Identification of Micro-organisms	12

CHAPTER 2: <u>EXAMINATION OF INDEPENDANT ISOLATES</u> <u>OF <i>Mycoplasma Ovipneumoniae</i> BY</u> <u>RESTRICTION ENDONUCLEASE ANALYSIS</u> <u>(REA)</u>	18
2.1 Introduction	18
2.2 Materials and Methods	18
2.21 Origin and Isolation of <i>M. ovipneumoniae</i>	18
2.22 Identification of the Mycoplasma isolates	19
2.23 Storage of stock culture of <i>M. ovipneumoniae</i>	19
2.24 Cloning of <i>M. ovipneumoniae</i>	20
2.25 Restriction Endonuclease Analysis (REA) of <i>M. ovipneumoniae</i>	20
a) Preparation of <i>M. ovipneumoniae</i> cells for DNA extraction	20
b) DNA Extraction	20
c) Estimation of the concentration of DNA in the extract	21
d) Spectrophotometry	22
e) Fluorimetry	22
f) Digestion of the DNA with <i>EcoRI</i>	23
g) Gel Electrophoresis of the DNA samples	25
h) Photography of the gel	26
2.26 Comparison of REA patterns	26
2.27 Gel precipitin tests	26
2.3 Results	28
2.31 Results of Identification tests	28
2.32 Results of REA of Independant isolates of <i>M. ovipneumoniae</i>	30
2.4 Discussion	37
CHAPTER 3: <u>STABILITY OF THE <i>M. ovipneumoniae</i> GENOME</u>	39
3.1 Serial passage of <i>M. ovipneumoniae</i> in vitro	39

3.11	Introduction	39
3.12	Materials and Methods	40
	a) Cloning of <i>M. ovipneumoniae</i> before serial passage.	40
	b) Analysis of the clone before passage	41
	c) First passage stage	41
	d) Labelling of cultures	41
	e) Second passage stage	42
	f) Third passage stage	42
3.13	Results	42
3.14	Conclusion	53
3.2	Reproducibility of REA pattern differences observed after the serial passage <i>in vitro</i> of <i>M. ovipneumoniae</i>	54
3.21	Introduction	54
3.22	Materials and Methods	54
	a) REA of duplicate cultures	54
	b) Effect of increased enzyme concentration on REA patterns	54
3.23	Results	55
	a) REA of duplicate cultures	55
	b) Effect of increased enzyme concentration on REA patterns	55
3.24	Conclusion	58
3.3	SDS Polyacrylamide gel electrophoresis (SDS-PAGE) of the proteins of <i>M. ovipneumoniae</i> clones which showed different REA patterns after serial passage <i>in vitro</i> .	59
3.31	Introduction	59
3.32	Materials and Methods	60
	a) Preparation of cell lysate	60
	b) Estimation of Protein content of <i>M. ovipneumoniae</i> lysates	60
	c) Preparation of the <i>M. ovipneumoniae</i> protein samples for SDS-PAGE Gel	61
	d) SDS-PAGE	61

3.33 Results	64
3.34 Conclusion	66
3.4 Discussion	67
CHAPTER 4: <u>GENETIC INTERCHANGE</u>	68
4.1 Introduction	68
4.2 "Classical" Antibiotic Resistance crosses	71
4.21 Introduction	71
4.22 Materials and Methods	71
a) Determination of Antibiotic concentrations for use in Recombination experiments	71
b) Selection of Antibiotic Resistant Mutants	71
c) "Classical" Antibiotic Resistance crosses	72
4.23 Results	73
a) Inhibitory concentrations of Naladixic Acid and Rifampicin	73
b) Selection of Antibiotic Resistant mutants	73
c) "Classical" Antibiotic Resistance crosses	73
4.24 Conclusion	75
4.3 REA of clones derived from mixed cultures	76
4.31 Introduction	76
4.32 Materials and Methods	76
a) REA of clones derived from a mixed culture of <i>M. ovipneumoniae</i> that had been passaged <i>in vitro</i> for five days.	76
b) REA of "presumptive recombinant" <i>M. ovipneumoniae</i> derived from "classical" antibiotic resistance crosses	76

4.33 Results	77
a) REA of clones derived from a mixed culture of <i>M. ovipneumoniae</i> that had been passaged <i>in vitro</i> for five days.	77
b) REA of "presumptive recombinant" <i>M. ovipneumoniae</i> derived from "classical" antibiotic resistance crosses.	79
4.34 Conclusion	82
4.4 Examination of <i>M. ovipneumoniae</i> for the presence of extrachromosomal DNA	83
4.41 Introduction	83
4.42 Materials and Methods	83
4.43 Results	84
4.44 Conclusion	86
4.5 Discussion	87
CHAPTER 5: <u>GENERAL DISCUSSION</u>	89
Appendix 1: <i>M. ovipneumoniae</i> growth and identification media	100
Appendix 2: REA materials	103
Appendix 3: SDS-PAGE Materials	106
Appendix 4: Plasmid identification materials	109
Bibliography	110

LIST OF FIGURES

FIGURE	Page
1. Standard Fluorimetry curve used to determine the DNA concentration present in <i>M. ovipneumoniae</i> extracts.	24
2. Gel precipitin test used in identification of Mycoplasma species.	29
3. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 1,2 and 3.	30
4. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 4, 5, and 6.	31
5. REA OF <i>M. ovipneumoniae</i> , isolates from CNP lesions of lambs from farms 7, 8 and 9.	32
6. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 10, 11 and 12.	33
7. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 13, 14 and 15.	34
8. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 16, 17 and 18.	35
9. REA of <i>M. ovipneumoniae</i> isolates from CNP lesions of lambs from farms 19 and 20.	36
10. REA of 8 clones of <i>M. ovipneumoniae</i> strain 5 selected simultaneously after 3 prior serial clonings.	44
11. REA of 8 clones selected after serial passage <i>in vitro</i> of a clone of <i>M. ovipneumoniae</i> strain	45

5 for approximately 133 cell generations.	45
12. REA of 8 clones selected simultaneously after serial passage <i>in vitro</i> of a clone of <i>M. ovipneumoniae</i> strain 5 for approximately 266 cell generations.	46
13. REA of 8 clones selected simultaneously after serial passage <i>in vitro</i> of a "variant" clone of <i>M. ovipneumoniae</i> strain 5 for approximately 133 cell generations.	47
14. REA of 8 clones selected simultaneously after serial passage <i>in vitro</i> of a clone of <i>M. ovipneumoniae</i> strain 5 for approximately 399 cell generations.	48
15. REA of 8 clones selected simultaneously after serial passage <i>in vitro</i> of a "variant" clone of <i>M. ovipneumoniae</i> strain 5 for approximately 133 cell generations.	49
16. REA of 8 clones selected simultaneously after serial passage <i>in vitro</i> of a "variant" clone of <i>M. ovipneumoniae</i> strain for approximately 266 cell generations.	50
17. REA showing the banding differences of which resulted in <i>M. ovipneumoniae</i> clones after serial passage <i>in vitro</i> .	51
18. A Flow diagram illustrating the serial <i>in vitro</i> passage of a <i>M. ovipneumoniae</i> strain 5 clone.	52
19. REA of duplicate cultures of <i>M. ovipneumoniae</i> clones that showed different REA patterns after serial passage <i>in vitro</i> .	56
20. REA of <i>M. ovipneumoniae</i> clones that showed	

different REA patterns after serial passage <i>in vitro</i> , digested with increased concentrations of <i>EcoRI</i> .	57
21. SDS-Polyacrylamide Gel Electrophoresis (SDS- PAGE) of <i>M. ovipneumoniae</i> clones that showed different REA patterns after serial passage <i>in</i> <i>vitro</i> .	65
22. REA of <i>M. ovipneumoniae</i> clones randomly selected from a mixed culture of <i>M. ovipneumoniae</i> strains 5 and 10.	78
23. REA of "presumptive recombinant" <i>M. ovipneumoniae</i> clones derived from "classical" antibiotic resistance crosses	80
24. REA of "presumptive recombinant" <i>M. ovipneu-</i> <i>moniae</i> clones derived from "classical" antibiotic resistance crosses.	81
25. Eckhardt plasmid gel of <i>M. ovipneumoniae</i> and <i>Rhizobium trifolii</i> isolates.	85

LIST OF TABLES

PAGE

Table I:	A Typical spectrophotometry profile for a nucleic acid sample extracted from <i>M. ovipneumoniae</i>	22
Table II:	Number of "presumptive recombinant" <i>M. ovipneumoniae</i> colonies observed on FM4 agar containing 4% of both Naladixic Acid and Rifampicin.	74
Table III:	Background level of Naladixic Acid resistant <i>M. ovipneumoniae</i> also resistant to Rifampicin.	74
Table IV:	Background level of Rifampicin resistant <i>M. ovipneumoniae</i> also resistant to Naladixic Acid.	74

Introduction

Chronic Non-Progressive pneumonia (CNP) is a disease which typically occurs in three to ten month old lambs. It has a complex aetiology, which has still not been unequivocally elucidated. However, it is apparent that *Mycoplasma ovipneumoniae* plays an important role in the pathogenesis of the disease. *M. ovipneumoniae* can be recovered from the nasal tract and lungs, and it is unclear if these represent a different population. Mew (1982) compared nasal and pneumonic strains of *M. ovipneumoniae* by Restriction Endonuclease analysis (REA) and from these studies it became apparent that many strains (as defined by their REA patterns) of *M. ovipneumoniae* could infect both the nasal cavities and lungs of lambs. To further investigate this heterogeneity of strains, Mew (1982) examined nine independent isolates (i.e. isolates which originated from different farms) by REA. All gave REA patterns that were completely different from each other. It therefore appeared that the species *M. ovipneumoniae* was extremely heterogeneous.

This heterogeneity contrasts with almost all other species examined by REA i.e. most microorganisms show either homogeneity e.g. *Brucella ovis* (O'hara *et al.*, 1984) or show limited heterogeneity e.g. *Campylobacter coli* (Kakoyiannis *et al.*, 1984). The present study therefore set out to further investigate the heterogeneity of *M. ovipneumoniae*.

To further examine the conclusion of Mew (1982), i.e. that the species *M. ovipneumoniae* shows considerable heterogeneity, a further sixty isolates of *M. ovipneumoniae*, i.e. three isolates from sheep on each of twenty farms. (thus giving a total of twenty independent isolates), were examined by REA. From this study it became apparent that considerable heterogeneity does exist in the species *M. ovipneumoniae*, so experiments were undertaken to answer the following questions:

1. Does genetic instability (i.e. rapid internal genetic change) in *M. ovipneumoniae* account for the heterogeneity

observed in the species?

This was investigated by analysis of *M. ovipneumoniae* clones before and after serial passage.

2. Does frequent genetic interchange in *M. ovipneumoniae* account for the heterogeneity observed in the species? (i.e. random genetic interchange between what were initially two strains of *M. ovipneumoniae* with markedly different genomes could generate an almost infinite number of strains which varied from their parental strains.

- This was investigated using three experimental approaches:

(a) "Classical" antibiotic resistance crosses, with selection for recombinants.

(b) REA of possible recombinants.

(c) Examination of *M. ovipneumoniae* for plasmids which, if present, might facilitate genetic interchange.

During the course of investigations into the genetic stability of *M. ovipneumoniae*, further questions arose concerning REA pattern changes:

1. Are the REA band changes observed real differences i.e. are they reproducible?

2. Do these changes in the REA patterns affect the antigenicity/protein component of the *M. ovipneumoniae* concerned?

These questions were also investigated.

3. If the strains are stable and independantly maintained, what is the minimum number of strains that is likely to be present and what is the likelihood that this number can be maintained?

Chapter 1. HISTORICAL REVIEW

1.1 Chronic Non-Progressive Pneumonia

1.11 Occurrence of Chronic Non-Progressive Pneumonia

Chronic Non-Progressive pneumonia (CNP) is enzootic in New Zealand sheep and typically occurs in 3 to 10 month old lambs. It differs from progressive pneumonia of sheep in that it normally resolves after a few months. Pathologically, CNP is characterised by varying degrees of dull red to grey consolidation of the anterior lobes. It causes less severe damage to the alveolar epithelium and the lesions are more localised than those in acute bacterial pneumonia.

A similar, probably identical, disease has been described in sheep in other countries including Australia (St George *et al.*, 1971), Great Britain (Jones *et al.*, 1975), The United States (St George and Carmichael, 1975), Iceland (Fris *et al.*, 1976) and Iraq (All-Sultan and Zubaidy, 1978).

A variety of names have been assigned to the disease: Summer pneumonia (St George *et al.*, 1972), Enzootic pneumonia (Kirton *et al.*, 1976), Atypical pneumonia (Stamp and Nisbet, 1963), Proliferative interstitial pneumonia (Carmichael *et al.*, 1972) and Chronic Non-Progressive Pneumonia (Alley, 1975). To avoid confusion, the name Chronic Non-Progressive Pneumonia (CNP) will be used in this thesis synonymously with the names mentioned above, as well as any other names used to refer to an apparently identical disease.

1.12 Economic Importance of Chronic Non-Progressive Pneumonia

It has been suggested that CNP is of great economic importance to New Zealand for two reasons

(1.) The formation of pleural adhesions and subsequent down-grading of such carcasses at the Meat works. (Dyshart, 1976). Pleurisy has been listed as the second most important (31.4%) disease/defect encountered at the Meat works. (Central Districts Farmer (1985) 3 (1))

(2.) Diminished Weight gains of lambs as a consequence of CNP

(St George *et al.*, 1971; Carmichael *et al.*, 1972; Harris and Alley, 1977; Jones *et al.*, 1982).

1.13 Aetiology of Chronic Non-Progressive Pneumonia

Although the disease referred to as CNP was described over two decades ago (Stamp and Nisbet, 1963), its aetiology has still not been unequivocally established.

Many microorganisms (*Mycoplasma ovipneumoniae*, *Mycoplasma arginini*, *Pasteurella haemolytica*, *Neisseria catarrhalis*, *Escherichia coli* and several viruses) have been recovered from the lung lesions, but not all have been consistently associated with the disease.

M. ovipneumoniae can be consistently isolated from the lesions, but may also be isolated from the lungs of apparently healthy sheep, although with decreased frequency and in lower titres (Alley, 1975; Davies *et al.*, 1981). Both *P. haemolytica* and *M. arginini* can be isolated from a smaller proportion of lung lesions than *M. ovipneumoniae*. (Alley, 1975; Jones *et al.*, 1978). *E. coli* and *N. catarrhalis* have only been isolated occasionally from CNP lesions (Alley, 1975; Alley and Clarke, 1979) but these two organisms are probably secondary invaders, as there is no other evidence of association with the disease. Several workers have suggested the Parainfluenza virus type III (PI3) may have a role in the aetiology of CNP eg. Jones *et al* (1979) detected a rise in antibody titre to PI3 in lambs which occurred concurrently with a rise in antibody titre to *M. ovipneumoniae* and the onset of a respiratory disease. However, these lambs were housed, so a rise in antibody titre to PI3, and no doubt a lot of other viruses could be hardly surprising with the stress and close contact imposed on the animals by the housing. Furthermore, PI3 has only been isolated once from lesions resembling those produced by CNP (Davies, 1980), other workers have failed to isolate PI3 from such lesions (St George *et al.*, 1971; Jones *et al.*, 1978, 1979; Brian, 1980).

Isolation alone of a microorganism from the lesions does

not imply causation of the disease. This, as Koch suggested in his postulates, requires transmission studies.

1.14 Transmission studies

Transmission studies have been carried out using different organisms and combination of organisms with CNP to try to elucidate the aetiology of the disease. These studies gave varying results and are summarised below.

Pneumonic Lung homogenate (This was derived from animals with CNP, and contained varying species and titres of the bacteria noted in the previous section). This was inoculated intranasally and produced a disease in over 50% of lambs. This disease resembled CNP (Jones *et al.*, 1978, 1980; Alley and Clarke, 1979, 1980; St George *et al.*, 1971). Transmission studies using lung homogenate as an inoculum allied with administration of antibiotics that inhibit either Mycoplasmas or (cell walled) bacteria significantly reduced the proportion of animals which consequently developed CNP (Alley and Clarke, 1980; Brian, 1980). This indicates that both a Mycoplasma and a (cell walled) bacteria are necessary to produce CNP.

Mycoplasma arginini cultures failed to produce CNP (Foggie and Angus, 1972). Furthermore, CNP has been produced with lung homogenate which did not contain *M. arginini* (Alley and Clarke, 1980).

Pasteurella haemolytica cultures inoculated into Specific Pathogen Free (SPF) lambs failed to produce CNP (Sharp *et al.*, 1978; Davies *et al.*, 1981).

Mycoplasma ovipneumoniae cultures inoculated into lambs have sometimes produced no disease (Davies *et al.*, 1981), but the majority of workers have produced a mild disease which resembled CNP. This disease occurred in a low proportion of the lambs.

Mixed Cultures Jones *et al.*, (1980), using an inoculum which

contained *M. ovipneumoniae*, *M. arginini*, and *P. haemolytica*, have produced a disease similar, if not identical, the CNP in a significant proportion of lambs. CNP has also been produced using a culture that contained *M. ovipneumoniae* and *P. haemolytica* but no *M. arginini* (Jones *et al.*, 1978; Gilmour *et al.*, 1982). Transmission studies so far used cloned cultures of *M. ovipneumoniae*. Thus, even though the isolate was derived from a CNP lesion, there is the possibility that it was one of many strains present in the lesions. These strains might differ in virulence or act in synergy. Jones *et al* (1982) found that an inoculum which contained *P. haemolytica* and a mixed culture of 6 strains of *M. ovipneumoniae* (all isolated from CNP lesions) produced the disease in a higher proportion of animals than when *P. haemolytica* was used in combination with a single strain of *M. ovipneumoniae*. Thus a method to differentiate between strains of *M. ovipneumoniae* was necessary to further elucidate the aetiology of CNP.

Mew (1982) developed such a method (based on REA) that differentiated between *M. ovipneumoniae* strains. Before the results of this work are discussed, it is first appropriate to discuss the background to Restriction Endonuclease analysis.

1.2 Restriction Endonucleases

1.21 Discovery and characterisation of restriction endonucleases

Luria *et al.*, (1952) were the first to describe the phenomenon that we now know as the Restriction and Modification system. They noticed that bacteriophages which had been propagated on one strain of bacteria displayed variation in their ability to propagate on other bacteria. This phenomenon was seen to be a property of the bacterial cells rather than the bacteriophages and thus led Luria *et al.* (1952) to postulate the existence of some type of cellular mechanism to account for this phenomenon.

Later studies by Arber and Dussoix (1962) on *Escherichia coli* strains B and K, led them to propose a model of restriction and modification to account for these observations. Thus they proposed that the bacterial cell contained two distinct enzymes, with paradoxical functions. One of these is an endonuclease (which recognises a specific sequence of bases within the DNA and consequently makes a double stranded cut of that DNA) and the other is a modification enzyme (which recognises the same sequence and makes a chemical alteration, eg. addition of a methyl group, to a specific site or sites within the recognition site, thus ensuring that it is no longer a substrate for the restriction enzyme). Thus the host (bacterial) DNA would always be modified, and hence protected from destruction by the Restriction enzyme. Any foreign DNA that invaded, provided it was unmodified, would therefore be destroyed.

Some years later the first Restriction endonucleases were isolated by Meselson and Yuan (1968) and Linn and Arber (1968) from *E. coli* K and B respectively. These enzymes (later designated class I restriction enzymes) recognise specific nucleotide sequences in the DNA, but cut at random sites away from the recognition sites. Two years later Smith and Wilcox (1970) and Kelly and Smith (1970) isolated an enzyme from *Haemophilus influenza*. This enzyme cut the DNA at the

recognition site, and is therefore referred to as a class II enzyme.

1.22 Class I Restriction Enzymes

Class I restriction enzymes are non specific with respect to their cutting sites. They require ATP, Mg^{2+} and S-adenosyl methionine. As proposed by Rosamund *et al* (1979) in a model of the action of *Eco* b, the enzyme binds to both the recognition sequence and some neighbouring bases in the presence of S-adenosyl methionine. While remaining bound to the recognition sequence, the other end of the enzyme utilises the energy from the ATP to form a gradually enlarging loop of DNA, by moving along the DNA to which it is bound. When the loop reaches a size between 1000 and 6000 base pairs, the enzyme produces a gap of about 75 nucleotides in one strand of the duplex. While the first enzyme continues to hold the DNA in the looped configuration, a second attaches and produces a cut in the remaining DNA strand. Thus a double stranded break in the DNA occurs at a site approximately 1000 nucleotides from the original recognition sequence. As Class I restriction enzymes are non-specific and hence produce heterogeneous products, their usefulness in genetic analyses is limited. Further discussion is therefore concerned mainly with class II Restriction Enzymes.

1.23 Class II Restriction Enzymes

Class II enzymes differ from Class I restriction enzymes in that they cut at a particular point in the recognition sequence. Class II enzymes are further divided into subgroups, based on the nature of the base sequence they recognise.

- (1) Symmetric i.e. The recognition sequence has 2-fold rotational symmetry and is thus a palindrome.

eg. *Eco*RI 5'GAATTC 3'
 3'CTTAAG 5'

- (2) Degenerate symmetric i.e. A portion of the recognition sequence forms a palindrome.

eg. *Hae*II 5'PuGCGPy3' Pu = Any Purine
 3'PyCGCPu5' Py = Any Pyrimidine

(3) Asymmetric i.e. No symmetry is seen in the recognition sequence.

eg. *Eco*PI 5'AGACC 3'
 3'TCTGG 5'

Class II restriction enzymes can produce 3 different types of fragments when they cut the DNA. If they cut in the middle of the recognition site (eg. *Bsu*RI which cuts thus 5'GG/CC3'), all the fragments produced have blunt ends. If they cut to one side of the middle of the recognition sequence, single stranded ends are produced. These ends can have either 5' tails (eg. *Bam* HI which cuts thus 5'G/GATCC 3') or 3' tails (eg. *Pst* I which cuts thus 5'CTGCA/G 3'). The production of these single stranded ends is useful in genetic studies, as will be discussed later (Although double stranded ends can be used in the same studies after modification).

1.24 Nomenclature of Restriction Enzymes

Restriction enzymes are designated by italicized letters representing the genus and the species name of the producing organism. A fourth letter may be added to indicate which specific strain produces the enzyme. When an organism produces more than one enzyme, the enzymes are differentiated by Roman numerals, eg. *Eco*RI is derived from *Escherichia coli* RY13.

About 200 Restriction enzymes have been discovered. (Restriction enzymes appear to be widespread throughout the bacterial kingdom, and each group of bacteria so far examined has produced at least one genus from which a restriction enzyme has been isolated.) As the recognition sequences for these enzymes are 4 to 7 bases long, enzymes which recognise the same sequence have been isolated from a variety of bacteria. These duplicate enzymes are known as isochizomers, and are still named according to the organism they are derived from.

1.25 Specificity of Class II Restriction Enzymes

Although the recognition sequence is necessary and sufficient for cleavage to occur, sequences outside this recognition sequence do influence the rate of cleavage. Furthermore specificity for the recognition sequence can be relaxed by changing the pH and ionic conditions of the reaction. Thus purification of the enzyme to Homogeneity is important. Most enzymes show a broad pH optimum, a broad magnesium optimum and are inhibited by high concentrations of sodium chloride. (Roberts, 1976).

1.26 Applications of Restriction Enzymes

Many applications of Restriction enzymes stem from their ability to produce small DNA fragments which can then be separated. The applications are in three main areas:

- (1) Physical Mapping of DNA.
- (2) Cloning of genes.
- (3) Classification and Identification of microorganisms
(and hence Epidemiological studies.)

Restriction Endonuclease mapping of DNA

Since class II restriction endonucleases are site specific, and thus produce a unique set of fragments from a particular DNA molecule, they can be used in the physical mapping of (viral and plasmid) DNA. These restriction maps are constructed by digestion of the DNA with a restriction endonuclease. The sizes of the fragments thus produced are then deduced by gel electrophoresis (The rate at which fragments move in the gel is a function of their size, with small fragments moving much faster than large fragments) and comparison with DNA fragments of a known size. The order of fragments in the DNA can then be deduced by studying the patterns of fragments produced as the digest proceeds to completion, and by redigestion of the fragments with another restriction endonuclease.

Thus a restriction map shows the positions of an endonucleases

cutting sites on the DNA. Each restriction endonuclease therefore produces a different restriction map for the same DNA molecule. These restriction maps are useful in the determination of regions of biological importance on the DNA.

Many restriction maps have been constructed since Darna and Nathans (1971) constructed the first restriction map (A *Hin* d II map of the circular DNA virus SV40). Thus restriction maps have been constructed for many viruses, including Adenoviruses (Tibbetts, 1977), Herpes Simplex virus type I (Skare and Summers, 1977) etc.

Cloning of genes

The realisation that many restriction endonucleases (eg *Eco* RI) generate specific cohesive ends (i.e. single stranded cohesive ends) that can be later sealed up by DNA ligase was followed by the development of the first practical method for cloning specific DNA fragments, regardless of their origin. The essential factor in the production of these cloned DNA fragments, is the random insertion of the restriction fragments of DNA molecule into a circular replicative plasmid DNA that had also been cut with the same endonuclease. These hybrid plasmids, which contain a section of DNA of interest, can then be used to infect bacteria. Thus each bacterial cell will acquire a recombinant plasmid carrying a specific piece of foreign DNA. Restriction endonucleases which do not produce single stranded cohesive ends can also be used to clone DNA fragments, once the blunt ended DNA fragments they produce have 'sticky ends' of single stranded DNA added to them.

The production of such recombinant DNA has produced almost endless possibilities in genetic manipulations, of which perhaps the most important are the possibilities that recombinant DNA opens up for the analysis of DNA from plants and animals.

Classification and Identification of Micro-organisms

Restriction Endonuclease Analysis (REA) i.e. digestion of the DNA with a restriction endonuclease, followed by gel electrophoresis of the fragments, is a relatively new technique. This technique was used initially with viruses to identify either species or individual strains. Thus species which are members of the Orthopoxviridae family i.e. rabbitpox, vaccinia, monkeypox, variola, cowpox and ectromelia all have REA patterns which are common within each species (Muller *et al.*, 1978; Esposito *et al.*, 1978; Mackett and Archard, 1979). Strains of Herpes simplex virus type 1 can also be distinguished by REA (Skare *et al.*, 1975). This has been used in epidemiological studies. Epidemiological studies using REA "fingerprinting" can in principle be applied to any DNA virus, but have so far been confined mainly to studies of Herpes Simplex virus types 1 and 2 (HSV-1 and HSV-2 respectively). Thus Buchman *et al* (1978) used REA to investigate a nosocomial outbreak of HSV-1, and showed that the outbreak was the result of two independent introductions of HSV-1. They therefore concluded that REA "has the potential of becoming a powerful tool for tracing the spread of HSV-1 and very likely of other herpesviruses in the human population." Other epidemiological studies using REA have also been carried out on HSV-1 (Linneman *et al.*, 1978; Lonsdale *et al.*, 1979).

REA studies can, in principle, be extended to all DNA viruses, since they have a genome size which limits the number of fragments, so that the resulting banding pattern shows good resolution and allows different banding patterns to be distinguished. The larger genome size of bacteria (giving a correspondingly greater number of fragments) seemed at first to preclude the use of REA in identification of bacteria, but in practice identification of strains, species and even genera of bacteria has proved possible.

Thus Marshall *et al* (1981) developed an REA technique that distinguished the *Leptospira interrogans* serovars *hardjo* and *balcanica* (which belong to the Hebdomadis serogroup - *Leptospira*

interrogans have at least 130 serovars which are grouped into 18 serogroups). These REA "fingerprints" were characteristic of the serovar, and no difference was seen between isolates derived from different host species or between isolates from the same host species from widely separated geographic areas in New Zealand. The REA "fingerprints" of the serovars *hardjo* and *balcanica* differed from other members of the same (*Hebdomadis*) serogroup and representatives of other *Leptospira interrogans* serogroups. Marshall *et al.*, (1981) therefore concluded that REA "should be useful for the identification of leptospires and might throw light on problems of their classification" and also suggested that the "the technique may prove useful in identifying bacteria other than leptospires." The latter statement has, indeed, proved to be true.

Thus Marshall *et al.* (1985) in a study of 94 isolates of *Moraxella bovis*, from the U.S.A., the U.K., Australia and New Zealand, were able to put the isolates into one of 26 groups on the basis of their REA patterns. These REA patterns were clearly different from prototype strains of other *Moraxella* species i.e. *Moraxella liquefaciens* and *Moraxella nonliquefaciens*.

Strains of *Campylobacter coli* have also been examined by REA. Thus Kakoyiannis *et al* (1984) examined 99 *C. coli* isolates derived from humans, pigs, gulls and chickens by REA. They found that the *C. coli* isolates from within these species could be grouped into REA groups. Thus the 14 human isolates examined produced 11 distinct REA types, the 43 pig isolates produced 20 REA types, the 10 gull isolates produced 5 different types, and the 32 chicken isolates produced 5 REA types. These REA types appear to be host specific e.g. None of the REA types which infected pigs were found in humans. (Note: one REA appeared to be non-host-specific, i.e. one human isolate was indistinguishable from a poultry isolate, however no direct epidemiological link between these isolates could be inferred however, as the isolates came from different areas). Some epidemiological observations can however be made from the results of Kakoyiannis *et al* (1984), apart from

the apparent host specificity e.g. Suckling piglets generally had identical *C.coli* REA types as their dams, and if they did vary, the *C.coli* REA type was similar to a type carried by a nearby sow to which the piglet had access. Thus Kakoyiannis *et al* (1984) concluded that REA "shows great promise for use in epidemiological studies of *C.coli*."

In some instances it is possible to use REA to distinguish a bacterial species and strains within the species. Thus Mielenz *et al* (1979) were able to distinguish the species *Rhizobium trifolii*, *Rhizobium meliloti*, and *Rhizobium japonicum* by their REA patterns. Strains within these species could also be detected by minor banding differences, on the species-specific background banding pattern. The value of REA in identification of *Rhizobium* species was highlighted by the identification of an isolate originally thought to be a *R.trifolii* mutant strain as a *R.japonicum* derivative. Mielenz *et al* (1979) summarised by suggesting that REA "will provide a useful tool for bacterial classification." The ability of REA to identify some genera supports this suggestion.

O'hara *et al* (1984) examined prototype species of *Brucella abortus*, *Brucella canis*, *Brucella melitensis* and *Brucella ovis* and found that they had between 80 and 90% of bands in common. So it appears that the genus *Brucella* can be identified by REA.

From the above review, it is apparent that REA can be used to identify bacterial strains, species, or even genera. Since this thesis is concerned with *M. ovipneumoniae*, we now review studies of the class Mollicutes by REA.

The class Mollicutes, with their genome of intermediate size between bacteria and viruses, have been extensively examined by REA. Thus species from the families Spiroplasmataceae, Acholeplasmataceae, and Mycoplasmataceae have all been examined.

The family Spiroplasmataceae consists of one genus, genus Spiroplasma, which in turn has two species within it.

(Bergey's Manual of Determinative Bacteriology, 1974).

The type species, *Spiroplasma citri* has been examined by Bove and Saillard (1979), who found that isolates of *S. citri* could be put into one of two groups, The first group consists of *S. citri* that have no extrachromosomal DNA and have identical REA patterns. The second group consists of *S. citri* that have varying complements of extrachromosomal DNA and hence has many REA patterns (which probably result from different complements of extrachromosomal DNA).

The family Acholeplasmataceae also consists of one genus, genus Acholeplasma within which there are 8 species. (Bergey's Manual of Determinative Bacteriology, 1974). Several of these have been examined by REA. For example Bove and Saillard (1979) showed that several different strains of *Acholeplasma laidlawii* were identical by REA. Similarly, Razin *et al* (1983a) found that the REA patterns of five *Acholeplasma oculi* strains showed only a few minor band difference, against a common background of bands. However, Razin *et al* (1983a) also found that eight *Acholeplasma axanthum* isolates (from widely diverse habitats) showed seven different REA patterns. Thus it seems that the species within the family may vary from homogeneity to great heterogeneity.

The family Mycoplasmataceae consists of two genera, genus Mycoplasma and genus Ureaplasma (Bergey's Manual of Determinative Bacteriology, 1974).

The solitary species of the genus Ureaplasma, *Ureaplasma urealyticum*, can be grouped into 2 distinct clusters by REA (Razin *et al.*, 1983b). Strains within a cluster differ by a few bands only. This led Razin *et al* (1983b) to suggest that the species *U. urealyticum* might represent two distinct species, as represented by the 2 distinct REA patterns.

The genus Mycoplasma consists of over sixty species. Of these, eight species (*Mycoplasma pneumoniae*, *Mycoplasma*

hypneumoniae, *Mycoplasma flocculare*, *Mycoplasma gallisepticum*, *Mycoplasma genitalium*, *Mycoplasma pulmonis*, *Mycoplasma hyorhinis* and *Mycoplasma ovipneumoniae*) have been examined by REA. Razin *et al* (1983a) examined three isolates of *M. genitalium*, two isolated from the urethra of patients with non-gonococcal urethritis and one from the urethra of an experimentally infected chimpanzee, and found that they had identical REA patterns. Similarly, Chandler *et al* (1982) and Razin *et al* (1983b) found that even though strains of *M. pneumoniae* may differ in their virulence and their adherence capabilities, they all have almost identical REA patterns. *M. gallisepticum* isolates have similarly been shown by Bove and Saillard (1979) and Razin *et al* (1983a) to have REA patterns very similar to each other, with at most only a few band differences. Two isolates of *M. pulmonis*, one a pathogen and the other not, were found by Bove and Saillard (1979) to be indistinguishable by REA. Chan and Ross (1984) have found that the species *M. flocculare* and *M. hyopneumoniae* are readily distinguishable by their REA patterns. Darai *et al* (1982) were even able to identify a *M. hyorhinis* cell culture contaminant by REA. The identity of the contaminant was later confirmed by classical means. This led Darai *et al* (1982) to suggest that "a more extensive use of restriction endonuclease analyses of different mycoplasma genomes ... will lead to a new basis for a more meaningful classification of mycoplasmas." Thus one might conclude from the above review that species of the genus *Mycoplasma* appear to be either homogeneous or show limited heterogeneity. However, when Mew (1982) examined *M. ovipneumoniae*, the results were remarkably different. Thus she (Mew, 1982) examined nine independent isolates of *M. ovipneumoniae* (i.e. isolates from sheep on different farms) by REA and all showed REA patterns that were completely different. Indeed, when *M. ovipneumoniae* isolates were taken from lambs on a single farm over a six month period, six more different strains were detected. Thus, this species shows considerable heterogeneity. This is in contrast to all other reports of species of the genus *Mycoplasma* examined by REA (which show either homogeneity or limited heterogeneity) and indeed almost all species of the class Mollicutes.

This thesis is thus concerned with the heterogeneity seen in *M. ovipneumoniae*.